EXHIBIT B

DiscoveRx

Homogeneous Assays for Tyrosine Kinase and Tyrosine Phosphatase Activity using β-Galactosidase Enzyme Fragment Complementation

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Abstract

Protein tyrosine kinases and phosphatases control several cellular responses including growth, proliferation and differentiation. We report a homogenous, non-isotopic assay using β-galactosidase EFC to detect inhibitors of tyrosine kinase and tyrosine phosphatase activity. In this format, inactive fragments of galactosidase, enzyme acceptor (EA) and enzyme donor (ED) complement to form active enzyme. Binding of an ED-conjugated peptide to an antibody inhibits complementation, while unlabeled peptide displaces the ED-conjugate. This results in increased β-galactosidase activity that is detected subsequently using either chemiluminescent or long wavelength fluorescent substrates.

HitHunter™ tyrosine kinase assay has been developed to measure activity of the human insulin receptor, EGF receptor kinase domains and Src (EC_{50} = 2.8 nM, 4.4 nM and 4.0 nM respectively). Hithunter™ tyrosine phosphatase activity was measured similarly using PTP 1B enzyme (EC_{50} = 48 nM). Assay performance characteristics (Z' = 0.5-0.7, CV = 5-8 %) and a simple two step addition protocol make it ideal for HTS.

Introduction

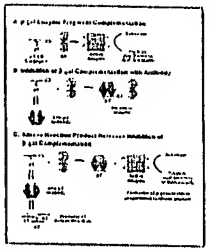
Tyrosine kinase (TK) plays a critical role in cellular signal transduction pathways involved in the regulation of many biological mechanisms. Protein phosphatase catalyzes the dephosphorylation of phosphoproteins; thus reversing the action of protein kinases. Protein phosphorylation and dephosphorylation are the basis for the regulation of many diverse cellular processes that include metabolism, cell division, differentiation and development. For this reason, tyrosine kinase and tyrosine phosphatase inhibitors are an important focus for new drug therapeutics.

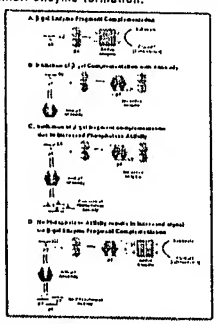
The HitHunter™ EFC Tyrosine Kinase and Tyrosine Phosphatase assays are robust, reliable assay platforms designed specifically for high-throughput screening. The EFC assay platform provides a rapid and homogeneous methodology for the detection of protein phosphorylation and dephosphorylation of tyrosine during the cell signaling process. The HitHunter™ EFC platform features the flexibility needed for high throughput screening with the following assay characteristics:

- Homogeneous
- Single well assay
- Single, biological label
- Miniaturizable (96/384/1536 formats)
- Luminescence or Fluorescence readouts
- Easily automated

HitHunter™ Enzyme Fragment Complementation

Enzyme Fragment Complementation Technology (EFC) is based on recombinant DNA techniques to genetically engineer β-galactosidase into two inactive fragments - Enzyme Acceptor (EA) and Enzyme Donor (ED). These fragments can recombine spontaneously to form active β-galactosidase by a process called complementation. The active tetrameric β-galactosidase can be measured by hydrolysis of fluorogenic or luminescent substrates. Attachment of a ligand to ED does not affect complementation, but subsequent binding of a receptor or binding protein will inhibit enzyme formation.





A Incubation of ED-pY conjugate with EA (Enzyme Acceptor) results in formation of active, tetramer β-galactosidase, which can be measured by hydrolysis of fluorogenic or luminescent substrates.

B Binding of anti-phosphotyrosine antibody to the ED-pY conjugate prevents formation of active enzyme.

C In the presence of kinase reaction products, competition for binding sites leads to an increase of free ED-pY ligand conjugate, which complements with EA to form active enzyme. Active enzyme hydrolyzes the substrate for readout either by luminescence or fluorescence. The amount of enzyme activity is directly proportional to the concentration of the kinase products and its relative affinity for the kinase substrate.

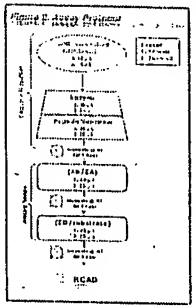
A Same as HitHunter™ EFC assay principle.

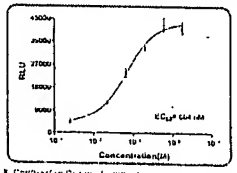
B Same as HitHunter™ EFC assay principle.

C Dephosphorylation of phosphotyrosines increases phosphatase products, leading to an increase of free ED-pY ligand conjugate. Binding of anti-phosphotyrosine antibody to ED-pY prevents formations of active enzyme. Lack of signal indicates increased phosphatase activity.

D In the presence of no phosphatase activity, phosphorylated tyrosines compete for binding sites. Thus, there is an increase in the amount of free ED-pY ligand conjugate, which complements with EA to form active enzyme. Active enzyme hydrolyzes the substrate for readout either by luminescence or fluorescence. The amount of enzyme activity is directly proportional to the concentration of the lack of phosphatase activity

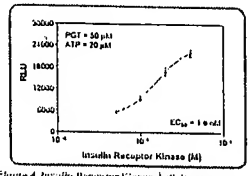
HitHunter™ EFC TK in a 384-well format



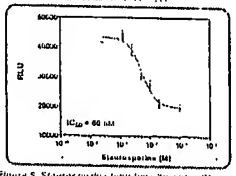


Tyrosine Kinase standard ranges from 0 to 18 µM in the 384-well assay. The standard curve illustrates a positive reading from low to high levels of phosphotyrosine.

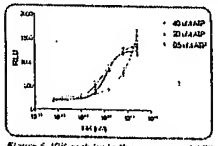
	0.69
	10
	0.66 µM/well



A concentration response curve for insulin receptor kinase enzyme was performed. The assay was initiated by the addition of ATP and PGT. The amount of phosphorylated peptide was quantitated after a two hour incubation at room temperature.

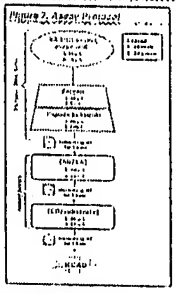


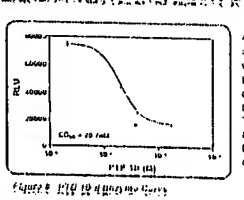
Staurosporine is a potent kinase inhibitor and was tested in the presence of IRK activity. HitHunter™ EFC Tyrosine Kinase Assay exhibited a dose dependent decrease in kinase activity in the presence of staurosporine.



Inhibitors of tyrosine kinase are often ATP competitors. The IRK enzyme activity was measured in the presence of varying concentrations of ATP. The data exhibits the utility of HitHunter™ EFC Tyrosine Kinase Assay over an ATP concentration range of 0.5 to 40 µM.

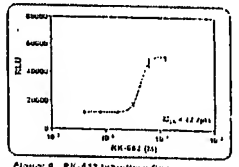
HitHunter™ EFC TR in a 384-well format





A concentration response curve for PTP 1B was performed under the established optimum conditions using PTP 1B substrate at 11.2 µM. The enzyme was tested at 2.5nM, 21 pM, 42 nM, 84 nM and 168 nM concentration

	0.72
	6
	0.66



RK-682 is a well known inhibitor of certain tyrosine phosphatases and was tested in the presence of PTP 1B activity (HitHunter™ EFC Tyrosine Phosphatase Assay) exhibited a dose dependent decrease in phosphatase activity in the presence of RK-682

Summary and Conclusion

SUMMARY

- HitHunter™ EFC assays for Tyrosine Kinase and Tyrosine Phosphatase have been demonstrated with both chemiluminescent and fluorescent substrates in a simple two step process that can be miniaturized in a 384 well plate format.
- Assay performance is excellent with Z' factor of 0.5-0.7 and CVs of 5-8%.
- Activity of human insulin receptor, EGF receptor kinase domains, and SRC can be measured with these assays.

CONCLUSION

The HitHunter™ EFC Tyrosine Kinase and Tyrosine Phosphatase Assays can detect phosphorylated and dephosphorylated tyrosines as a result of enzyme reactions with no wash / transfer steps and is assayed in the same well, making it highly suitable for HTS screening for kinase and phosphatase inhibitors.